REMARKS

Status of the Claims

Claims 5, 7, 13, 15, 19, 21, 27, 29, 33, and 35 have been amended. Specifically, claims 5, 13, 19, 27, and 33 have been amended to recite only glycosylated forms of IFN-β, while claims 7, 15, 21, 29, and 35 have been amended to recite the version of the ALIGN program (*i.e.*, version 2.0). Support for the latter amendment can be found throughout the specification, for example on page 9, lines 4-7. No new matter is added by way of claim amendments.

Claims 1-35 are pending in the application. Reexamination and reconsideration of the claims is respectfully requested in view of these amendments and the following remarks. The Examiner's comments in the Office Action are addressed below in the order set forth therein.

The Objection to Claims 5, 6, 13, 14, 19, 20, 27, 28, 33, and 34 Should Be Withdrawn

Claims 5, 6, 13, 14, 19, 20, 27, 28, 33, and 34 are objected to under 37 CFR 1.75(c) as being in improper dependent form for allegedly failing to further limit the subject matter of a previous claim. As amended, claims 5, 13, 19, 27, and 33 further limit interferon-beta (IFN-β) to glycosylated IFN-β, while claims 6, 14, 20, 28, and 34 further limit IFN-β to recombinantly produced IFN-β.

As set forth in 37 CFR 1.75(c), "[o]ne or more claims may be presented in dependent form, referring back to and further limiting another claim or claims in the same application." Such further limitation can be by addition of an element to a claim, and/or by adding a limitation to a previously recited element. Applicants respectfully submit that the recited limitations "glycosylated" (for claims 5, 13, 19, 27, and 33) and "recombinantly produced" (for claims 6, 14, 20, 28, and 34) further limit a previously recited element (*i.e.*, IFN-β), and therefore claims 5, 6, 13, 14, 19, 20, 27, 28, 33, and 34 are in proper dependent form and this objection should be withdrawn.

The Rejection of the Claims Under 35 U.S.C. §112, First Paragraph, Should Be Withdrawn

Enablement

Claims 1-35 are rejected under 35 U.S.C. §112, first paragraph, for allegedly failing to comply with the enablement requirement of Section 112. Specifically, the Examiner asserts that the specification, while being enabling for mature, native IFN-β (SEQ ID NO:1) or a variant thereof (SEQ ID NO:2), does not provide guidance or examples showing any other variant, fragment or truncated IFN-β, or any polypeptide with less than 100% sequence identity to SEQ ID NO:1, that could be prepared by the claimed methods and still retain any biological function. This rejection is respectfully traversed.

The present invention is drawn to methods for preparing IFN-β, including denaturing IFN-β by dissolving IFN-β in guanidine HCl followed by renaturation via dilution into a first buffer. In some embodiments the renatured IFN-β is substantially monomeric. In still other embodiments, the renatured IFN-β is diafiltered or dialyzed into a second buffer to remove residual guanidine HCl.

As an initial matter, Applicants note that original claims 4, 12, 18, 26, and 32 do not recite variants, fragments, truncations, or partial structures with percent identity to SEQ ID NO:1, but rather recite SEQ ID NO:1 or SEQ ID NO:2. Consequently, Applicants submit that claims 4, 12, 18, 26, and 32 should not have been rejected for allegedly failing to comply with the enablement requirement using the arguments provided by the Examiner.

In rejecting claims 1-35 for allegedly failing to comply with the enablement requirement of Section 112, the Examiner contends that "there is no guidance or examples showing any other variant, fragment, or truncated IFN-β, or any polypeptide with less than 100% sequence identity to SEQ ID NO:1, that could be prepared by the claimed methods and still retain any biological function which would provide utility to the injectable formulation" (Office Action mailed October 5, 2006, page3, lines 29-32). Applicants respectfully disagree.

To satisfy the enablement requirement of 35 U.S.C. § 112, first paragraph, the specification must teach those skilled in the art to make and use the full scope of the claimed invention without undue experimentation. *Enzo Biochem, Inc. v. Calgene, Inc.*, 188 F.3d 1362, 1371, 52 USPQ2d 1129, 1135 (Fed. Cir. 1999); *Genentech, Inc. v. Novo Nordisk, A/S*, 108 F.3d

1361, 1365, 42 USPQ2d 1001, 1004 (Fed. Cir. 1997); *PPG Inds., Inc. v. Guardian Inds. Corp.*, 75 F.3d 1558, 1564, 37 USPQ2d 1618, 1623 (Fed. Cir. 1996); *In re Wright*, 999 F.2d 1557, 1561-62, 27 USPQ2d 1510, 1513 (Fed. Cir. 1993); *In re Vaeck*, 947 F.2d 488, 495-96, 20 USPQ2d 1438, 1444-45 (Fed. Cir. 1991). "That some experimentation may be required is not fatal, the issue is whether the amount of experimentation required is 'undue.'" *In re Vaeck*, 947 F.2d at 495, 20 USPQ2d at 1444. The enablement section of 35 U.S.C. § 112, first paragraph, "requires that the scope of the claims must bear a reasonable correlation to the scope of enablement provided by the specification to persons of ordinary skill in the art." *In re Fisher*, 427 F.2d 833, 839, 166 USPQ 18, 24 (C.C.P.A. 1970). In order to determine whether the present claims are enabled, an analysis of the teachings of the specification must be performed as well as an inquiry into the knowledge of persons of ordinary skill in the art. *In re Bowen*, 492 F.2d 859, 861, 181 USPQ 48, 50 (C.C.P.A. 1974).

As a member of a well-characterized protein family, variants of IFN-β were well known to those of skill in the art at the time the present application was filed. As stated in the specification on page 10, lines 9-17:

> Non-limiting examples of IFN-β polypeptides and IFN-β variant polypeptides encompassed by the invention are set forth in Nagata *et al.* (1980) *Nature* 284:316-320; Goeddel *et al.* (1980) *Nature* 287:411-416; Yelverton *et al.* (1981) *Nucleic Acids Res.* 9:731-741; Streuli *et al.* (1981) *Proc. Natl. Acad. Sci. USA* 78:2848-2852; EP028033B1, and EP109748B1. See also U.S. Patent Nos. 4,518,584; 4,569,908; 4,588,585; 4,738,844 [*modified beta interferons containing amino acid substitutions in amino acids 1 to 28*]; 4,753,795 [*modified beta interferons containing amino acid substitutions in amino acids 80 to 113*]; 4,769,233 [*modified beta interferons containing amino acid substitutions in amino acids 17 and 32-52*]; 4,793,995 [*modified beta interferons containing amino acid substitutions in amino acids 1 to 56*]; 4,914,033; 4,959,314; 5,545,723 [*an IFN-β mutein in which phe, tyr, trp, or his is substituted for val at position 101*]; and 5,814,485. These disclosures are herein incorporated by reference. These citations also provide guidance regarding residues and regions of the IFN-β polypeptide that can be altered without the loss of biological activity.

Furthermore, assays to determine whether IFN-β variants encompassed by the invention retain biological activity (*e.g.*, the ability to bind to IFN-β receptors) are routine to one of skill in

the art. See, for example, the extensive listing of references found in the specification on page 10, lines 1-8, all of which were well known in the art prior to the filing of the present application. Applicant reminds the Examiner that "[a] patent need not teach, and preferably omits, what is well known in the art." Manual of Patent Examining Procedure (MPEP) §2164.01, citing to *In re Buchner*, 929 F.2d 660, 661, 18 USPQ2d 1331, 1332 (Fed. Cir. 1991); *Hybritech, Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1384, 231 USPQ 81, 94 (Fed. Cir. 1986), *cert. denied*, 480 U.S. 947 (1987).

The Examiner cites Mickle *et al.* (*Med. Clin. North Am.* 84:597-607, 2000) for the proposition that "even the substitution or deletion of a single amino acid can have dramatic and *unpredictable* effects on the function of a protein. Due to this uncertainty, a person of ordinary skill in the art would not be able to predict which amino acid residues, or polypeptide regions/domains, could be altered and still retain biological activity" (Office Action, page 4, lines 12-15; emphasis in original). The Examiner cites this reference as evidence of undue experimentation on the part of the skilled artisan (Office Action, page 4, line 16). The Examiner, however, fails to consider the entire teachings of the Mickle *et al.* reference relative to IFN-β variants.

First, Mickle *et al.* discuss genotype-phenotype relationships in cystic fibrosis (CF). The CF protein is a massive polypeptide of 1,408 amino acids. The CF protein is in no way related to the much smaller (*i.e.*, 166 amino acids) IFN-β of the present invention, and the cited reference relating to CF does not bear any relevance to IFN-β. Second, contrary to the Examiner's assertions, as discussed above a person of ordinary skill in the art would be able to predict which amino acid residues, or polypeptide regions/domains, of IFN-β could be altered and still retain biological activity, as IFN-β is a member of a well-characterized protein family. Thus, the teachings of Mickle *et al.* do not support the Examiner's conclusion that the present claims lack enablement.

In the present case, all the Examiner has established is that some experimentation would be required to make and use certain embodiments of the claimed invention (*e.g.*, injectable formulations of IFN-β including IFN-β with less than 100% sequence identity to SEQ ID NO:1). What the Examiner has not done is perform the fact-finding needed in order to reach a proper

conclusion of undue experimentation. The Examiner has not relied upon any evidence in support of this rejection which would establish that methods for preparing an injectable formulation of IFN-β including other sequences beyond those described in the present specification amounts to undue experimentation. In fact, the Examiner has ignored the guidance in the specification and the teachings of the prior art and makes the rejection based upon unsupported opinions.

Applicants respectfully submit that there is a vast amount of supporting evidence in the field of IFN-β molecular biology, which is also supported by the disclosure of the present application, on how to routinely make and use biologically active variants of IFN-β. Therefore, the enablement rejection should be withdrawn.

Written Description

Claims 1-35 are rejected under 35 U.S.C. §112, first paragraph, for allegedly failing to comply with the written description requirement of Section 112. Specifically, the Examiner maintains that the claims contain subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the art that the inventor had possession of the claimed invention at the time the application was filed. This rejection is respectfully traversed.

The "Guidelines for Examination of Patent Applications Under the 35 U.S.C. 112, 1, 'Written Description' Requirement" state that a genus may be described by "sufficient description of a representative number of species . . . or by disclosure of relevant, identifying characteristics, i.e. structure or other physical and/or chemical properties." 66 Fed. Reg. 1106 (January 5, 2001). This is in accordance with the standard for written description set forth in *Regents of the University of California v. Eli Lilly & Co.*, 119 F.3d 1559 (Fed. Cir. 1997), where the court held that "[a] written description of an invention involving a chemical genus, like a description of a chemical species, 'requires a precise definition, such as by structure, formula, or chemical name' of the claimed subject matter sufficient to distinguish it from other materials." 119 F.3d at 1568, citing *Fiers v. Revel*, 984 F.2d 1164 (Fed. Cir. 1993).

The Federal Circuit has made it clear that sufficient written description requires simply the knowledge and level of skill in the art to permit one of skill to immediately envision the

product claimed from the disclosure. *Purdue Pharma L.P. v. Faulding Inc.*, 230 F.3d 1320 1323, 56 USPQ2d 1481, 1483 (Fed. Cir. 2000) ("One skilled in the art must immediately discern the limitations at issue in the claims."). In the instant case, the Examiner asserts that "[a]lthough the specification does describe some examples of IFN-β polypeptides that can be used in the claimed method, these examples are insufficient to define the genus of IFN-β polypeptides, which can encompass any variant, fragment, or truncated IFN-β polypeptide. Thus, the claims are drawn to a genus of polypeptides that has not been adequately described in the specification" (Office action, page 5, lines 3-7). Applicants respectfully disagree.

As discussed above, Applicants note that original claims 4, 12, 18, 26, and 32 do not recite variants, fragments, truncations, or partial structures with percent identity to SEQ ID NO:1, but rather recite SEQ ID NO:1 or SEQ ID NO:2. Consequently, Applicants submit that claims 4, 12, 18, 26, and 32 should not have been rejected for allegedly failing to comply with the written description requirement using the arguments provided by the Examiner.

A satisfactory disclosure of a "representative number" of species depends on whether one of skill in the art would recognize that the applicants were in possession of the necessary common attributes or features of the elements possessed by the members of the genus in view of the species disclosed. 66 Fed. Reg. 1099, 1106 (2000). Applicants submit that the knowledge and level of skill in the art would allow a person of ordinary skill to envision the claimed invention, that is, injectable formulations of IFN-β including IFN-β with less than 100% sequence identity to SEQ ID NO:1. As described above, as a member of a well-characterized protein family, variants of IFN-β were well known to those of skill in the art at the time the present application was filed. Furthermore, as also described above, assays to determine whether IFN-β variants encompassed by the invention retain biological activity (*e.g.*, the ability to bind to IFN-β receptors) are routine to one of skill in the art and were well known in the art prior to the filing of the present application.

Applicants respectfully submit that sufficient written description is provided to reasonably convey to one skilled in the relevant art that the inventors, at the time the application was filed, had possession of the claimed invention. Accordingly, the written description rejection should be withdrawn.

The Rejection of the Claims Under 35 U.S.C. §112, Second Paragraph, Should Be Withdrawn

Claims 7, 15, 21, 29, and 35 are rejected under 35 U.S.C. §112, second paragraph, as being indefinite for allegedly failing to point out and distinctly claim the subject matter which Applicants regard as the invention. Specifically, the Examiner asserts that it is not clear which version/year of the ALIGN program must be used, and therefore the claims are indefinite. Applicants have amended claims 7, 15, 21, 29, and 35 to recite version 2.0 of the ALIGN program. Accordingly, reconsideration and withdrawal of this rejection are respectfully requested.

The Rejection of the Claims Under 35 U.S.C. §103 Should Be Withdrawn

Claims 8-35 are rejected under 35 U.S.C. §103(a) for allegedly being unpatentable over Arora *et al.* (*J. Biotech.* 52:127-33, 1996) in view of Dorin *et al.* (U.S. Patent No. 5,814,485). This rejection is respectfully traversed.

Arora *et al.* teach a method for isolating IFN-γ from *E. coli* inclusion bodies, including solubilization of the inclusion bodies in guanidine HCl, renaturation of the solubilized protein mixture by dilution in a refolding buffer, dialysis of the renatured protein mixture against a second buffer, and purification of the IFN-γ following loading of the resulting solution on a S-Sepharose column. Dorin *et al.* teach methods of recombinant production of IFN-β and its subsequent purification, and disclose various excipients that can be included in IFN-β formulations having a pH of about 6.0 to 7.5.

The Examiner contends that motivation to combine Arora *et al.* and Dorin *et al.* "comes from the teaching of Arora *et al.*, which shows the IFN polypeptides can be purified and properly folded by first dissolving the polypeptides in guanidine, followed by dilution into a buffer to renature the IFN polypeptides, and finally, dialysis to remove the guanidine. Further motivation comes from the teachings of Dorin *et al.*, which teaches an IFN-β polypeptide with 100% sequence identity to SEQ ID NO:1 of the instant application, and the usefulness of this polypeptide in the treatment of human disease" (Office Action, page 6, line 32 through page 7, line 4). Applicants respectfully disagree.

It is well settled in the case law that obviousness can only be established by combining or modifying the teachings of the prior art to produce the claimed invention where there is some suggestion or motivation to do so found either explicitly or implicitly in the references themselves or in the knowledge generally available to one of ordinary skill in the art. "The test for an implicit showing is what the combined teachings, knowledge of one of ordinary skill in the art, and the nature of the problem to be solved as a whole would have suggested to those of ordinary skill in the art." *In re Kotzab*, 217 F.3d 1365, 1370, 55 USPQ2d 1313, 1317 (Fed. Cir. 2000). The mere fact that references can be combined or modified does not render the resultant combination obvious unless the prior art also suggests the desirability of the combination. *In re Mills*, 916 F.2d 680, 16 USPQ2d 1430 (Fed. Cir. 1990).

In this case, Applicants submit that, contrary to the Examiner's assertions, nothing in Arora *et al.* or Dorin *et al.* would have motivated the skilled artisan to combine their teachings to arrive at Applicants' methods for preparing IFN-β. As discussed above, Arora *et al.* teach a method for isolating **IFN-γ** from inclusion bodies. It is well known to one of ordinary skill in the art that multiple factors affect isolation and purification of recombinant proteins, each dependent on the nature of the protein itself (see, *e.g.*, Fiona *et al.* in *Guide to Protein Purification*, ed. by Deutscher, MP., Ch. 20, Academic Press, Inc., 1990, pp. 264-76; attached as **Exhibit A**). At most one of skill in the art would view the combination of these two references as an invitation to experiment with multiple parameters (*e.g.*, concentrations of the solubilizing agent and buffers, temperature and pH) to ascertain their suitability in methods for preparing **IFN-β**. Yet an invitation to experiment is not sufficient grounds to reject an invitation as obvious. One of skill in the art at the time of the invention would not have had sufficient guidance to have a reasonable expectation of success in combining the teachings of Arora *et al.* with Dorin *et al.* to arrive at Applicants' claimed invention. Where the prior art gives only general guidance as to the particular form of the invention or how to achieve it, as here, obviousness may not be found. *Hybritech, Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 231 USPQ 81, 90-91 (Fed. Cir. 1986).

Claims 1-7 are rejected under 35 U.S.C. §103(a) for allegedly being unpatentable over van Oss (*J. Protein Chem.* 8:661-68) in view of Arora *et al.* (*J. Biotech.* 52:127-33, 1996) and further in view of Dorin *et al.* (U.S. Patent No. 5,814,485). This rejection is respectfully traversed.

van Oss discusses the well known technique of protein precipitation with ethanol. As discussed above, Arora *et al.* teach a method for isolating IFN-γ from inclusion bodies and Dorin *et al.* teach methods of recombinant production of IFN-β and its subsequent purification. The Examiner alleges that "[b]y teaching that ethanol precipitation of proteins is a common and effective procedure for protein isolation, van Oss provides the motivation to incorporate ethanol precipitation of IFN-β into the method taught by the combination of Arora *et al.* and Dorin *et al.* as described (Office Action, page 7, line 29 through page 8, line 1).

The Federal Circuit has expressly stated that "[o]bviousness cannot be established by combining the teachings of the prior art to produce the claimed invention, absent some teaching suggestion or incentive supporting the combination." *In re Geiger*, 2 USPQ2d 1276, 1278 (Fed. Cir. 1987). Applicants submit that nothing in van Oss remedies the lack of motivation to combine Arora *et al.* and Dorin *et al.*, as discussed above. The mere fact that the different elements of an invention may be disclosed in the prior art is insufficient to establish obviousness without a motivation to combine the prior art references. Accordingly, a showing by van Oss that ethanol precipitation of proteins is a common and effective procedure for protein isolation is insufficient to render the present claims obvious. As noted by the Federal Circuit:

> Most if not all inventions arise from a combination of old elements. Thus, every element of a claimed invention may often be found in the prior art. However, identification in the prior art of each individual part claimed is insufficient to defeat the patentability of the whole claimed invention. Rather, to establish obviousness based on a combination of the elements disclosed in the prior art, there must be some motivation, suggestion or teaching of the desirability of making the specific combination that was made by the applicant. *In re Kotzab* 55 USPQ2d 1313, 1316 (Fed. Cir. 2000) (internal citations omitted).

In the present case, the cited references lack any suggestion or motivation to combine their teachings to arrive at the methods for preparing IFN-β discovered by Applicants. As

discussed above, the combination of the references at best is an invitation to one of skill in the art to perform additional experiments. However, an invitation to experiment is not sufficient grounds to reject an invitation as obvious.

Furthermore, the Examiner has failed to provide evidence beyond broad conclusory statements that combining the references is appropriate. See *Ex parte Skinner*, 2 USPQ2d 1788, 1790 (B.P.A.I. 1986) (stating that "it is the duty of the examiner to explain why combination of the reference teachings is proper"). Given the lack of evidence of a motivation to combine the references, it appears that the Examiner has engaged in impermissible "hindsight reconstruction" in formulating the present rejection. See *In re Fine*, 5 USPQ2d 1071, 1075 (Fed. Cir. 1988) (holding that "[o]ne cannot use hindsight reconstruction to pick and choose among disclosures in the prior art to deprecate the claimed invention"). In establishing obviousness, it is improper "to use the claimed invention as an instruction manual or template to piece together the teachings of the prior art so that the claimed invention is rendered obvious." *In re Fritch*, 23 USPQ2d 1780, 1784 (Fed. Cir. 1992).

In view of the above arguments, Applicants contend that a *prima facie* case of obviousness under 35 U.S.C. §103(a) has not been established. Accordingly, reconsideration and withdrawal of the rejections are respectfully requested.

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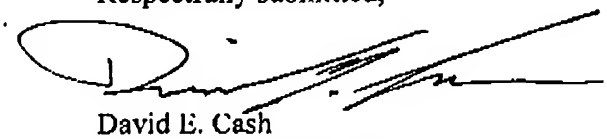
CONCLUSION

In view of the aforementioned amendments and remarks, Applicants respectfully submit that the rejections of the claims under 35 U.S.C. §§112 and 103 are overcome. Applicants respectfully submit that this application is now in condition for allowance. Early notice to this effect is solicited. If in the opinion of the Examiner, a telephone conference would expedite the prosecution of the subject Application, the Examiner is invited to call the undersigned attorney.

It is not believed that extensions of time or fees for net addition of claims are required, beyond those that may otherwise be provided for in documents accompanying this paper. However, in the event that additional extensions of time are necessary to allow consideration of this paper, such extensions are hereby petitioned under 37 CFR § 1.136(a), and any fee required therefore (including fees for net addition of claims) is hereby authorized to be charged to Deposit Account No. 16-0605.

Respectfully submitted,



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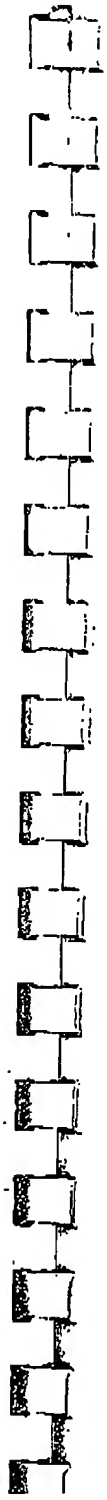
Nora C. Martinez

APPENDIX A

Appl. No.:	10/750,076	Docket No.:	PP16022.017(35784/271881)
Applicant(s):	Wolfe et al.	Customer No.:	45853
Filed:	December 31, 2003		
Confirmation No.:	2260		
Art Unit:	1646		
Examiner:	Bruce D. Hissong		
Title:	METHODS OF PROTEIN PURIFICATION AND RECOVERY		



Methods in Enzymology

Volume 182

Guide to Protein Purification

EDITED BY

Murray P. Deutscher

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4. Solubilization of Protein. Experimental points should be determined in triplicate. An aliquot of 0.5 ml of the stock membrane suspension is pipetted into each tube at 4°. An aliquot of 0.5 ml of the differing detergent concentrations at twice their final concentration to be tested is then pipetted into appropriate tubes. Tubes are maintained at 4° for 1 hr, and then centrifuged at 105,000 *g* for 1 hr at 4°. Tubes may be stirred during the incubation period, but excessive agitation should be avoided since foam formation is associated with the denaturation of proteins.

5. Determination of Solubilized Protein. After centrifugation, the clear supernatant should be removed from any residual pellet. Protein determinations should be made both on the solubilized protein and any pellet after suspension in an equal volume of solubilization buffer. Some pellets are difficult to resuspend, and may require the use of a small tissue homogenizer. Aliquots of the solubilized protein and the resuspended pellet can be assayed for the individual protein or activity being studied.

Conclusion

The discussion presented here provides a few relatively simple guidelines for the selection of detergents and their use in the solubilization of membrane proteins in a native state. This is truly a task about which few valid generalizations may be made. For this reason, general recipes concerning the total amount of detergent to be used, or specific suggestions of "nondenaturing" detergents which will be useful in all situations, cannot be made.

[20] Solubilization of Protein Aggregates

By FIONA A. O. MARSTON and DONNA L. HARTLEY

Introduction

Major advances in genetic engineering have resulted in the development of bacterial cell systems, especially *Escherichia coli*, capable of producing high levels of proteins expressed from cloned genes.[1,2] In *E. coli*, in a majority of cases, the overexpressed protein accumulates intracellularly in an insoluble form, resulting in phase-bright inclusions in

[1] J. F. Kane and D. L. Hartley, *Trends Biotechnol.* 6, 95 (1988).
[2] F. A. O. Marston, *Biochem. J.* 240, 1 (1986).

the cytoplasm.[3-5] Solubilization o
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From a purification standpoir
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General Considerations

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due to a high localized co

[3] D. C. Williams, R. M. Van Fr
[4] R. G. Schoner, L. F. Ellis, a
[5] L. A. Holladay, R. G. Hamn
[6] D. F. Marks, S. D. Lu, A. A
Sci. U.S.A. 81, 5662 (1984).
[7] D. L. Hartley and J. F. Kan
[8] W. F. Prouty, M. J. Karnov
[9] C. A. Schuchtele, D. L. An
[10] C. K. Tuggle and J. A. Fuc

[20] SOLUBILIZATION OF PROTEIN AGGREGATES 265

the cytoplasm.[3-5] Solubilization of these protein aggregates is the subject of this chapter.

From a purification standpoint, the accumulation of protein in an aggregated form is advantageous. After breaking open the cells and centrifuging the resulting lysate the aggregated protein can be recovered in the pellet fraction about 50% pure, although mostly in an inactive form. The protein in the inclusion bodies can be a mixture of monomeric and multimeric forms,[6] both reduced and oxidized.[2] The major problem then becomes one of recovering biologically active protein in high yield. In order to accomplish this the protein in the inclusion bodies must be solubilized, refolded, and purified, in a specific order.[2]

The common stages in processes designed to recover biologically active, soluble protein from such aggregates include (1) cell lysis, (2) isolation of inclusion bodies, (3) solubilization of protein in inclusion bodies, and (4) refolding of solubilized protein.

In the following sections we first present general approaches used to isolate and solubilize protein aggregates. Upon refolding such methods may yield active soluble protein; however, there are many factors influencing the final yield, each dependent on the nature of the protein itself. The later sections of the chapter illustrate these factors by reference to the processes optimized for specific proteins.

General Considerations

Protein inclusion bodies in *E. coli* are formed during high-level expression of cloned genes.[1,2] Exactly why inclusion bodies form is not known, but there are parallels between such cells and cells in which the heat-shock system has been induced.[7-9] The majority of protein contained within these inclusion bodies is in a denatured form, in part due to the reducing environment of the *E. coli* cytoplasm.[10] In addition, dimers and higher molecular weight multimers may be present. Hydrophobic interactions between regions of the unfolded protein molecules in the inclusion bodies may also be important. That the protein is not simply precipitated due to a high localized concentration is evidenced by the fact that strong

[1] D. C. Williams, R. M. Van Frank, W. L. Muth, and J. P. Burnett. *Science* **215**, 687 (1982).
[4] R. G. Schoner, L. F. Ellis, and B. E. Schoner, *Bio/Technology* **3**, 151 (1985).
[5] L. A. Holladay, R. G. Hammons, Jr., and D. Puett, *Biochemistry* **13**, 1653 (1974).
[6] D. F. Marks, S. D. Lu, A. A. Creasey, R. Yamamoto, and L. S. Lin, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 5662 (1984).
[7] D. L. Hartley and J. F. Kane, *Biochem. Soc. Trans.* **16**, 101 (1988).
[8] W. F. Prouty, M. J. Karnovsky, and A. L. Goldberg, *J. Biol. Chem.* **250**, 1112 (1975).
[9] C. A. Schachtele, D. L. Anderson, and P. Rogers, *J. Mol. Biol.* **33**, 861 (1968).
[10] C. K. Tuggle and J. A. Fuchs, *J. Bacteriol.* **162**, 448 (1985).

TABLE I
REAGENTS THAT RELEASE EUKARYOTIC POLYPEPTIDES FROM INCLUSION BODIES INTO SOLUTION[a]

Reagent	Eukaryotic polypeptide solubilized
Guanidine-HCl (5–8 *M*)	Insulin A and B chains
	Bovine growth hormone (bGH)
	Urokinase
Urea (6–8 *M*)	Prochymosin
	Interferon
	Salmon growth hormone
SDS	Interferon
	Interleukin 2 (IL-2)
Alkaline pH (>9.0)	Prochymosin
	Chicken growth hormone
Acetonitrile/propanol	T4 *regA* protein

[a] Reproduced from Ref. 21.

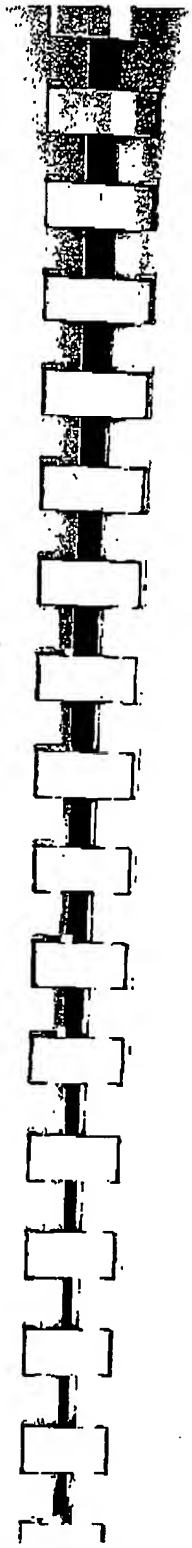
"solvents" are necessary to solubilize the proteins,[2] as illustrated in Table I.

Once proteins are solubilized, the task becomes one of refolding the protein molecules into their correct conformations. This can be accomplished by removing the solubilizing agent and replacing it with an aqueous buffer (e.g., by dialysis or dilution). The situation is somewhat more complicated in proteins containing disulfide bridges. Oxidation of the reduced protein must occur either prior to or concomitant with refolding.

The concentration of protein in the refolding solution also affects the yield of recoverable active protein.[11] The most significant loss during refolding of concentrated protein solutions is due to aggregate formation, which is frequently due to covalent modifications of the unfolded protein molecules, such as intermolecular disulfide formation.[11] It is therefore recommended that refolding be carried out at as dilute a concentration as feasible, taking into consideration workable volumes of solutions and possible loss due to high dilution for subsequent purification steps.

Finally, purification is usually effected to remove other proteins and contaminating nucleic acids also present in the inclusion body,[7] as well as to remove incorrectly refolded forms of the protein of interest. Depending on the specific characteristics of the protein to be purified, conditions can often be defined so that an ion-exchange column can effectively remove

[11] A. Light, *Bio/Technology* 3, 298 (1985).



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[12] Y. Saito, Y. Is
[13] D. N. Brems, S
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[14] S. M. Zimowsk
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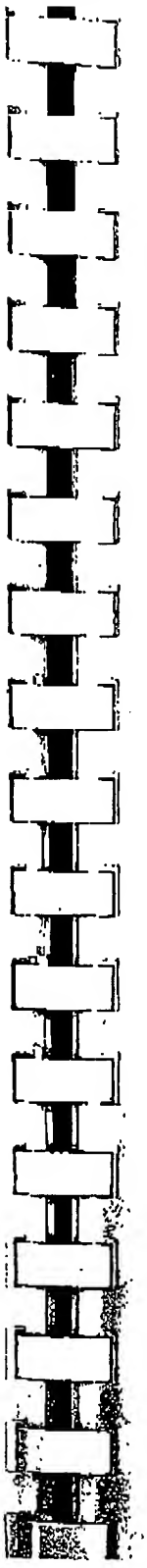
[15] J. Bartholomé-
Maruyama, H.
[16] D. V. Goeddel,
Hirose, A. Kra
106 (1979).
[17] G. Schumacher
[18] J. A. Goliger a



nucleic acid contaminants and proteins of very different isoelectric points. Subsequent purification is frequently carried out by HPLC.[2,12] With very low level expression, i.e., very small inclusion bodies, more impurities will be present than when starting with large inclusion bodies,[13] and will undoubtedly have a negative impact on the efficiency of solubization and refolding. This emphasizes the importance of optimizing expression either by genetic manipulations or by amelioration of fermentation and induction parameters, before attempting to purify significant quantities of the expressed protein.

General Solubilization Scheme

Preparation of Certain Solubilization Reagents

Urea (6 M). Ultrapure-grade urea should be used, with deionized distilled water. This solution should be deionized by passage through a mixed bed ion-exchange resin and stored at 4°. Cold storage will reduce the formation of cyanate ions, which can ultimately react with amino groups to form carbamylated derivatives.

Guanidine-HCl (6 M). Ultrapure grade guanidine is used and the pH of the final solution is adjusted to 7–8 with concentrated HCl.

Cell Disruption

There are several ways to recover protein inclusion bodies from pelleted bacterial cells. The most frequently used are sonication[12,14,15] and passage through a French pressure cell,[16,17] but it is also possible to use the lysozyme–detergent treatment.[18]

Sonication. Suspend 15 g of cell paste in 50 ml of chilled deionized water. Sonicate two times for 45 sec each at 0°, using 50% power. Keep cell suspension on ice.

[12] Y. Saito, Y. Ishi, M. Niwa, and I. Ueda, *J. Biochem. (Tokyo)* **101**, 1281 (1987).
[13] D. N. Brems, S. M. Plaisted, H. A. Havel, E. W. Kauffman, J. D. Stodola, L. C. Eaton, and R. D. White, *Biochemistry* **24**, 7662 (1985).
[14] S. M. Zurawski, T. R. Mosmann, A. Benedik, and G. Zurawski, *J. Immunol.* **137**, 3354 (1987).
[15] J. Bartholomé-DeBelder, M. Nguyen-Distèche, N. Houba-Hérin, J. M. Ghuysen, I. N. Maruyama, H. Hara, Y. Hirota, and M. Inouye, *Mol. Microbiol.* **2**, 519 (1988).
[16] D. V. Goeddel, D. G. Kleid, F. Bolivar, H. L. Heyneker, D. G. Yansura, R. Crea, T. Hirose, A. Kraszewski, A. Itakura, and A. D. Riggs, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 106 (1979).
[17] G. Schumacher, D. Sizmann, H. Haag, and A. Bock, *Nucleic Acids Res.* **14**, 5713 (1986).
[18] J. A. Goliger and J. W. Roberts, *J. Biol. Chem.* **262**, 11721 (1987).

French Press. Prechill the French press cell to be used. Suspend 15 g of cell paste in 30 ml of chilled deionized water. Pass the cell suspension through the cell twice at 5000 lb/in. pressure. Keep the suspension on ice.

Centrifugation and Washing

Centrifuge the suspension resulting from cell breakage at 10,000 to 20,000 *g* for 10 to 20 min at 4°. (At this point it is prudent to look at a sample of the supernatant under the microscope to determine if a significant quantity of inclusion bodies remain. This can vary depending on the protein being expressed by the cells. If significant amounts of inclusion bodies remain in the supernatant, recentrifuge at 20,000–25,000 *g* for 10–15 min.) Carefully decant the supernatant and resuspend the pellet in 50 ml of chilled deionized water.

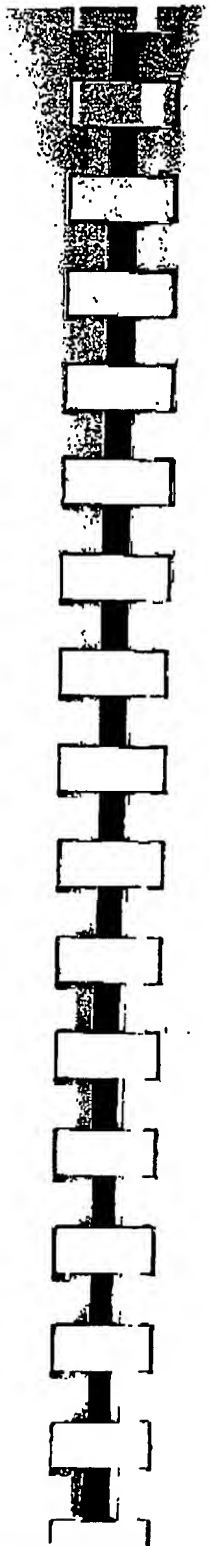
Washing Inclusion Bodies

The fact that many of the eukaryotic proteins expressed in *E. coli* are insoluble is an advantage since isolation of inclusion bodies in itself can be a very efficient purification step. However, to a varying degree, protein contaminants do remain after inclusion body isolation by centrifugation coupled with washing procedures. Such contaminants may subsequently interfere with refolding or may prevent it altogether. Most, if not all, of these contaminating proteins are strongly associated with or entrapped in the inclusion bodies and therefore may require solubilization under the type of conditions listed in Table I to release them.

It is frequently advantageous to wash the inclusion bodies with a solution other than water. In this case, the wash step can be carried out as above, usually with the incorporation of some type of detergent in the washing buffer.[15,19] It has also been found that treatment of the broken cell pellet with 0.2 mg ml^{-1} lysozyme/1 m*M* EDTA/1 mg ml^{-1} deoxycholate, while the protein is still insoluble, will remove the majority of nucleic acids, phospholipids, and lipopolysaccharides from bovine somatotropin inclusion bodies, without solubilizing the protein.[19] A similar wash, using lysozyme/EDTA/DNase/Nonidet was also found to be effective in selectively removing contaminants without solubilizing the protein in the inclusion bodies.[15]

Denaturants can in some cases be used to solubilize contaminants preferentially. Directly expressed bovine growth hormone (bGH) forms inclusion bodies in *E. coli*, and a number of contaminants copurify with

[19] K. E. Langley, T. G. Berg, T. W. Strickland, D. M. Fenton, T. C. Boone, and J. Wypych, *Eur. J. Biochem.* 163, 313 (1987).



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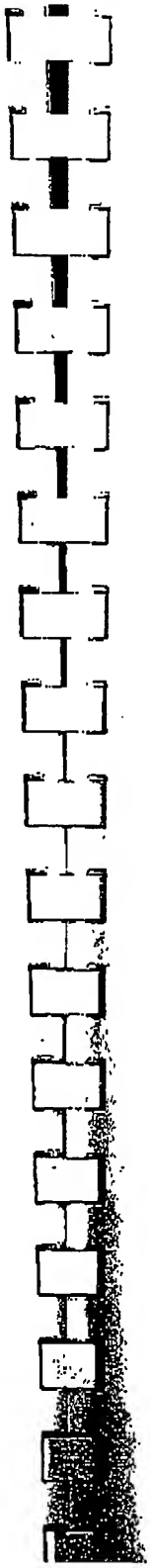


the aggregated bGH.[4] Washing the inclusion bodies with up to 4 *M* urea only solubilizes the contaminants, but as the urea concentration is increased to 5 *M* the bGH is partially solubilized as well.

Solubilization

Typical classes of reagents which can be used to solubilize proteins from inclusion bodies are listed in Table I. In general a solubilization and refolding protocol for protein aggregates in *E. coli* will be protein specific. At each stage there are variables which are critical and must be considered. For the solubilization stage these variables are as follow:

1. pH
2. Incubation temperature
3. Time of exposure to solvent
4. Ionic components of the solvent
5. Concentration of the solubilization agent
6. Concentration of total protein
7. Ratio of solubilization agent to protein
8. Presence or absence of redox agents
9. Derivatization of thiol groups

Having established the optimum reagent for solubilization, the other variables listed must be investigated in conjunction with refolding to determine the most efficient overall process. A recommended strategy is to screen the variables first on a small scale (1- to 2-ml working volume) in order to select a limited number of variables to evaluate on a larger scale.

Commonly denaturants are used early in the evaluation of solubilization agents. A typical method might be as follows:

Using a clean spatula, remove the washed inclusion body paste to a preweighed glass beaker and determine its weight. Add a stir bar and use a magnetic stir plate on the lowest speed possible. Slowly add a solution of 6 *M* urea or 6 *M* guanidine chloride to give a final protein concentration of 1–2 mg/ml. Continue stirring at the lowest speed until the paste is dissolved. It is frequently desirable to add a thiol reagent, such as cysteine or 2-mercaptoethanol, to the urea or guanidine-HCl solution to reduce all of the protein present in the inclusion bodies.

For example, it has been estimated that only 80% of the bGH in inclusions is in a reduced form.[19] In order to recover active protein from the remaining oxidized material, it is necessary to reduce it, thus starting with 100% reduced material (i.e., random coil). In contrast, solubilization

of prochymosin (in urea or guanidine-HCl) in the presence of thiol reagents has a negative impact on the recovery of active protein.[20]

If the solution resulting from solubilization is quite cloudy, it is recommended that a centrifugation step be added here. Centrifuge the protein solution at 20,000–30,000 *g* for 15 min. Decant the cleared supernatant to a fresh beaker.

Refolding

Protein aggregates in *E. coli* must be first solubilized and then refolded in order to regain active protein. These two processes, solubilization and refolding, are interdependent and their use in recovering proteins from aggregates in *E. coli* has been reviewed.[2,21] As for solubilization, there are variables at the refolding stage which are critical and must be considered:

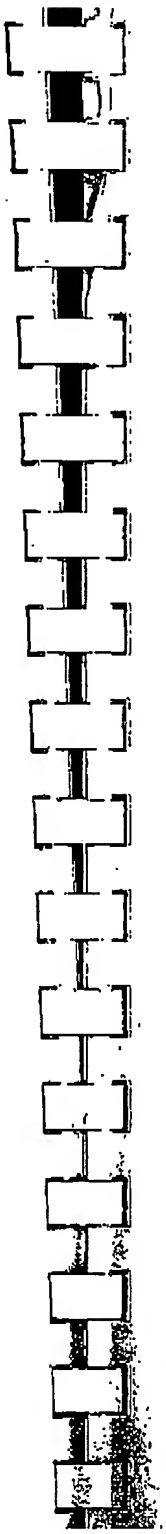
1. pH
2. Incubation temperature
3. Time
4. Ionic components of the solvent
5. Rate of change from solubilization to refolding solvent conditions
6. Purity of the protein of interest
7. Concentration of the protein of interest
8. Presence or absence of redox agents

It is pertinent in this chapter to consider the key points relating to solubilization which impact on refolding.

It is important during both solubilization and refolding to minimize exposure to conditions which result in derivatization of amino acid side chains (e.g., pH values of greater than 9.0). During the refolding stage perhaps the key parameters influencing recovery are purity and concentration of the protein of interest. The concentration of the protein should be such that at the incubation temperature used, intramolecular bonds form in preference to intermolecular bonds.

Finally, for proteins which contain cysteine residues and in their native form contain disulfide bonds, the redox conditions during solubilization and refolding may be critical. Strict guidelines cannot be provided since requirements are protein specific. Prochymosin, for example, contains six cysteine residues which form three disulfide bonds in the native protein. If aggregated, prochymosin isolated from *E. coli* is first fully

[20] F. A. O. Marston, P. A. Lowe, M. T. Doel, J. M. Schoemaker, S. White, and S. Angal, *BioTechnology* 2, 800 (1984).
[21] F. A. O. Marston, *in* "DNA Cloning" (D. Glover, ed.), Vol. 3, p. 59. IRL Press, Oxford, 1987.



reduced, and then solubilized in a strong denaturant (8 *M* urea). However, little if any activity is recovered upon refolding. The best yields of active protein could be obtained by omitting redox reagents at both the solubilization and refolding stages.[20]

In contrast, there are examples of proteins which require redox reagents at the solubilization stage, or refolding stage, or both. One such example is interleukin 2 (IL-2) (three cysteine, one disulfide) which is in a fully reduced form in inclusion bodies.[22] At the solubilization stage it apparently is essential to maintain the denatured IL-2 in a reduced form.[23] In both this study and another in parallel,[22] it was then clearly demonstrated that oxidative refolding is required to obtain biologically active IL-2.

Solubilization of Hybrid Protein Aggregates

Recombinant DNA technology can be used to produce hybrid proteins by fusion of the coding sequence of the gene of interest to one or more other gene sequences. This is a common strategy used to improve expression levels. The mechanism by which expression levels are increased can differ and may be the result of reduced proteolysis[24] or sequestration of the gene product into inclusion bodies.[25]

Cleavage of the hybrid protein *in vitro* may be required in order to generate the protein of interest. The cleavage strategy must be considered in advance of gene construction and an appropriate cleavage site engineered immediately before the N-terminus (or after C-terminus) of the coding sequence of the protein of interest. Such a cleavage site must either be absent in the required protein or, if present, must be less reactive than the sequence located at the junction. Essentially, there are two methods of cleavage which can be employed: chemical or enzymatic. When the hybrid protein is aggregated and insoluble such cleavage must be performed under conditions which solubilize the protein and make the cleavage site accessible.

Extreme acid conditions can solubilize polypeptides and concomitantly catalyze hydrolysis. Conditions can be adjusted such that hydrolysis occurs at specific amino acid sequences, typically after Asp. For native proteins partial acid hydrolysis is typically performed in 30 m*M* HCl,

[22] T. Tsuji, R. Nakagawa, N. Sugimoto, and K.-I. Fukuhara, *Biochemistry* **26**, 3129 (1987).
[23] M. P. Weir and J. Sparks, *Biochem. J.* **245**, 85 (1987).
[24] K. Itakura, T. Hirose, R. Crea, A. D. Riggs, H. L. Heyneker, F. Bolivar, and H. W. Boyer, *Science* **198**, 1056 (1977).
[25] S.-H. Shen, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 4627 (1984).

TABLE II
CONDITIONS USED FOR CNBr CLEAVAGE OF CERTAIN AGGREGATED HYBRID PROTEINS

Hybrid protein	Percentage formic acid (v/v)	CNBr : protein (w/w)	Incubation time (hr)	Protein concentration (mg ml^{-1})	Ref.
Tandem-linked proinsulin or β-galactosidase–proinsulin	70	50	35	—	25
beZ–substance P	88	10	12	15.0	29
β-galactosidase–growth hormone	44	5	3	20.0	30

in vacuo at 105° for 20 hr.[26] As an example, acid hydrolysis was used to release bGH from fusions to the *trpE* and *trpLE* gene products.[27]

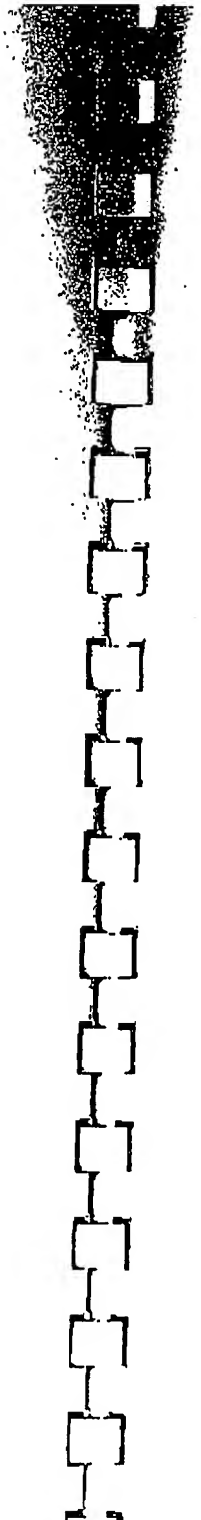
However, acid conditions alone were not adequate to solubilize aggregated proteins for cleavage. The suspending medium required was 70% formic acid containing the denaturant guanidine hydrochloride at a concentration of 6 *M*.[27] The proteins were incubated at concentrations of between 0.85 and 1.0 mg ml^{-1} for 72 hr at 37° to allow cleavage to occur.

When the protein of interest lacks Met residues in its sequence, a common strategy is to construct the fusion protein with a Met residue at the junction between the two parts of the hybrid molecule. Then cyanogen bromide (CNBr), which selectively cleaves proteins immediately after Met residues, can be used to cleave the hybrid *in vitro*. CNBr cleavage is performed under acid conditions (commonly formic acid), and therefore aggregated proteins may be solubilized.[28]

Key parameters to consider when establishing hydrolysis conditions include protein concentration, formic acid concentration, ratio of CNBr to protein (w/w), temperature, and time. Table II[29,30] illustrates, with a few examples of insoluble hybrid proteins, the wide range of conditions which have been employed. In each of these examples, the acid conditions alone were adequate for the CNBr to gain access to the cleavage site.

When cleavage of hybrid proteins is to be catalyzed enzymatically, the

[26] R. L. Lundblad and C. M. Noyes, *in* "Chemical Reagents for Protein Modification," Vol. I. CRC Press, Boca Raton, Florida, 1984.
[27] P. R. Szoka, A. B. Schreiber, H. Chan, and J. Murphy, *DNA* 5, 11 (1986).
[28] E. Gross, this series, Vol. 11, p. 27.
[29] T. Kempe, S. B. H. Kent, F. Chow, S. M. Peterson, W. I. Sundquist, J. J. L'Italien, D. Harbrecht, D. Plunkett, and W. J. DeLorbe, *Gene* 39, 239 (1985).
[30] T. Kempe, F. Chow, S. M. Peterson, P. Baker, W. Hays, G. Opperman, J. J. L'Italien, G. Long, and B. Paulson, *Bio/Technology* 4, 565 (1986).



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hybrid should be purified to >80% homogeneity (see following section) to maximize the efficiency of the proteolytic step. There are two possible proteolytic strategies for aggregated hybrids:

1. Solubilize the hybrid proteins and cleave in the presence of the solubilization agent.
2. Refold the solubilized hybrid, removing the solubilization agent, and then cleave.

Strategy (1) involves the use of balanced conditions which are severe enough to dissociate aggregates but which do not inactivate the proteolytic enzyme. There are certain proteases which are active in the presence of high levels of denaturant, e.g., clostripain (4 *M* urea[31]) and carboxypeptidase (5 *M* urea[32]). In an example of this strategy fusion between the bacterial enzyme chloramphenicol acetyltransferase (CAT) and human calcitonin (hCT) was engineered with a -Lys-Arg- cleavage site,[31] which can be cleaved by clostripain. The hybrid CAT-Lys-Arg-hCT was purified by isolation and washing of inclusion bodies. The washed inclusions were then solubilized in 100 m*M* Tris-HCl, pH 7.8, containing 8 *M* urea and 0.14 *M* 2-mercaptoethanol at a final protein concentration of 40 mg ml^{-1}. The suspension was incubated at 37° for 10 min and then diluted 1 : 1 (v/v) with water. Clostripain was then added to a final ratio of protease : fusion protein (w/w) of 1 : 40 and the suspension was incubated for a further 15 min at 37° before the addition of trifluoroacetic acid [5% (v/v), final concentration] to stop the enzymatic reaction.

With strategy (2), in which hybrid proteins are solubilized and refolded before cleavage, the potential problem is that the components of the hybrid may interact and therefore prevent correct folding. However, the strategy has been used successfully with fusions between the λ*cII* gene product and β-globin.[33] The fusion protein is isolated in washed inclusion bodies that were solubilized in 8 *M* urea and purified (see below). Then dialysis is used to remove the denaturant and allow refolding. Cleavage of the fusion protein is then catalyzed with blood coagulation factor Xa, approximately 160 mg of fusion protein being cleaved with 5 mg of activated factor Xa.

Purification of Solubilized Protein Aggregates

Once the typical washing procedures described earlier have been used effectively to purify inclusion bodies, the only method to dissociate the

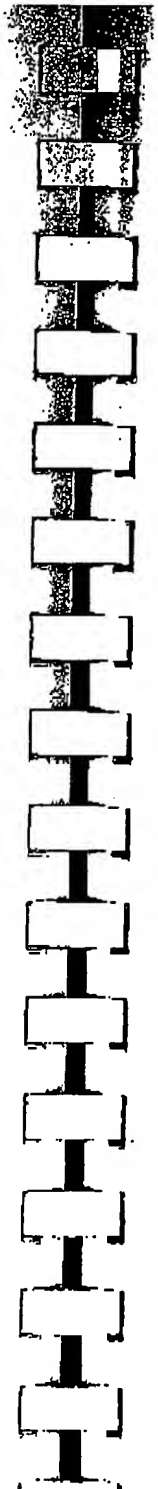
[31] P. A. Lowe, S. K. Rhind, R. Sugrue, and F. A. O. Marston, *Protein Purif.: Micro Macro–UCLA Symp. Mol. Cell. Biol.* 68, 429 (1987).
[32] H. M. Sussenfeld and S. J. Brewer, *BioTechnology* 2, 76 (1984).
[33] K. Nagai, M. F. Perutz, and C. Poyart, *Proc. Natl. Acad. Sci. U.S.A.* 82, 7252 (1985).

TABLE III
CHEMICAL STABILITY OF SELECTED CHROMATOGRAPHY MATRICES

Chromatographic mode	Matrix	Stability			Supplier
		pH	Denaturants	Detergent	
Ion exchange	Mono-S	2–12	8 *M* urea	Nonionic, anionic, zwitterionic	Pharmacia
Ion exchange	Mono-Q	2–12	8 *M* urea	Nonionic, cationic, zwitterionic	Pharmacia
Ion exchange	TSK-DEAE 5PW	2–12	8 *M* urea, 6 *M* GuHCl	All	Toyo-Soda
Ion exchange	DEAE-Sephacel	2–12	8 *M* urea, 6 *M* GuHCl	Nonionic, anionic	Pharmacia
Gel filtration	Sepharose CL	3–14	8 *M* urea, 6 *M* GuHCl	All (sodium deoxycholate not recommended)	Pharmacia
Gel filtration	Superose	2–12	8 *M* urea, 6 *M* GuHCl	All (sodium deoxycholate not recommended)	Pharmacia
Hydrophobic interaction	Phenyl-Superose	2–12	8 *M* urea	Nonionic, cationic, zwitterionic	Pharmacia
Hydrophobic interaction	TSK Phenyl 5PW	2–12	8 *M* urea, 6 *M* GuHCl	All	Toyo-Soda
Reversed phase	PLRP-S	1–13	Urea, GuHCl	All	Polymer Laboratories
Reversed phase	Ultrapore short chain C3	2.5–7.5	Urea, GuHCl	All	Beckman

remaining contaminating proteins may be total solubilization using the harsh conditions listed in Table I. However, it is still possible to purify the protein of interest using conventional chromatography matrices, which are stable under such extreme conditions (Table III).

The protocol developed to solubilize the λ*cII*–β-globin fusion protein from *E. coli*[33] involves isolation and washing of inclusion bodies, solubilization in 8 *M* urea, and purification of the denatured protein before refolding and cleavage of the hybrid protein. Isolated inclusion bodies were solubilized in 8 *M* urea, 25 m*M* Tris-acetate, pH 5.0, 1 m*M* EDTA, and 1 m*M* DTT (urea buffer). The solubilized protein was applied to a CM-Sepharose column and the fusion protein eluted with a gradient of 0–0.2 *M* NaCl in urea buffer. The eluted protein was then subjected to gel



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filtration on a Sephacryl S-200 column in 5 *M* guanidine-HCl, 5 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA, 1 m*M* DTT. The eluate from this column containing the fusion protein was dialyzed to remove denaturant and allow the hybrid protein to refold before cleavage with factor Xa as described in an earlier section.

In *E. coli* over 10 eukaryotic proteins have been expressed fused to λ*cII* via the factor Xa cleavage site.[34] In each case the process sequence was similar: solubilization (denaturation), purification in a denatured state, refolding, and cleavage. The level of purity required for efficient refolding differed and was found to be protein specific.

Interleukin 2 (IL-2) in *E. coli* is another example of an aggregated protein that has been purified in a solubilized, denatured form before refolding.[23] IL-2 contains three cysteine residues and the intramolecular disulfide bond between Cys-58 and Cys-105 in native IL-2 is essential for activity. Partial purification of the solubilized IL-2 facilitated analysis of reoxidation of the molecule during refolding and therefore allowed optimization of refolding conditions to give the maximum yield of correctly oxidized IL-2. Purification was effected by gel filtration in 6 *M* guanidine-HCl, 50 m*M* Tris-HCl, pH 8.5, 10 m*M* DTT using Superose-12 (FPLC) on an analytical scale and Sepharose CL-6B on a larger scale.

One final purification strategy to consider is that in which gene fusions are designed and constructed to facilitate purification. The principle of this strategy is to fuse the gene of interest to a sequence coding for a polypeptide which is selectively recognized by a matrix-bound ligand. A general approach developed by Sassenfeld and co-workers[32] was to produce C-terminal polyarginine fusions. Efficient purification of such fusions is possible using two-step cation-exchange chromatography. For example, urogastrone–polyarginine[32] expressed in *E. coli* is insoluble and can be solubilized by sonication of whole cells in 5 *M* urea, 40 m*M* Tris–acetate, NaOH, pH 9.5. The supernatant from centrifugation of such an extract, adjusted to pH 5.5, was subjected to chromatography on SP-Sephadex. The fusion protein and other cationic proteins bound and were eluted with a 0–300 m*M* NaCl gradient. After digestion of the polyarginine tail with carboxypeptidase B, urogastrone does not bind, while the other cationic proteins do. Therefore, the second cation-exchange step yields highly pure urogastrone. The buffer used for both cation-exchange steps was 5 *M* urea, 40 m*M* Tris–acetate, pH 5.5.

There are examples of this fusion protein purification strategy which predate the polyarginine fusion approach (e.g., β-galactosidase[35] and

[34] K. Nagai, H.-C. Thorgensen, and B. F. Luisi, *Biochem. Soc. Trans.* 16, 108 (1988).
[35] M. Koenen, U. Ruther, and B. Muller-Hill, *EMBO J.* 1, 509 (1982).

CAT[36]). However, the conformation of these polypeptide components of the hybrid are essential for selective recognition by the affinity ligand. Therefore, when CAT–hCT was found to be insoluble in *E. coli* and required 8 *M* urea to effect solubilization, affinity chromatography was not possible.[31]

There have been recent developments in the design of fusion proteins which facilitate purification utilizing maltose-binding protein[37] and glutathione *S*-transferase.[38] However, a central aim of each of these strategies is to obtain a fusion protein which is soluble in the cytoplasm or is secreted to the periplasm.

Concluding Remarks

The protocols described in this chapter illustrate the solubilization and refolding requirements of specific proteins. Purification and analysis of the proteins during and after these processes are of importance but are not considered here as they are discussed in detail in later chapters in this volume.

However, it is important to emphasize that certain features of the final protein products may be the direct results either of the expression mechanism or the methods used to recover active, soluble protein. These include

1. Authenticity of the N-terminus/C-terminus
2. Lack of posttranslational modification (e.g., glycosylation)
3. Modifications resulting from solubilization conditions (e.g., deamidation at high pH, Met oxidation, and conversion of cysteine to cysteic acid at low pH)
4. Conformational authenticity of the refolded molecule

These features will undoubtedly affect the activity of the final product, and their impact will depend on the application for which the proteins are required.

[36] A. D. Bennet, S. K. Rhind, P. A. Lowe, and C. C. G. Hentschel, U.K. Pat. No. GB 2140810 B (1983).
[37] C. di Guan, P. Li, P. D. Riggs, and H. Inouye, *Gene* 67, 21 (1988).
[38] D. B. Smith and K. S. Johnson, *Gene* 67, 31 (1988).